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Please, provide the following. Thank you!

1) Wang et al, Virology, 1992, Vol. 223, pp. 41-50.

2) Lu et al, Virology, 1995, Vol. 214, pp. 222-228.

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## Binding of the Influenza Virus NS1 Protein to Double-Stranded RNA Inhibits the Activation of the Protein Kinase That Phosphorylates the eIF-2 Translation Initiation Factor

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The NS1 protein of influenza A virus binds not only to poly(A) and a stem-bulge region in U6 small nuclear RNA (snRNA), but also to double-stranded (ds) RNA. Binding assays with NS1 protein mutants established that the previously identified RNA-binding domain of the NS1 protein is required for binding to ds RNA as well as for binding to poly(A) and U6 snRNA. In addition, dsRNA competed with U6 snRNA for binding to the NS1 protein, consistent with both RNAs sharing the same binding site on the protein. As a consequence of its binding to dsRNA, the NS1 protein blocks the activation of the dsRNA-activated protein kinase (PKR) *in vitro*. This kinase phosphorylates the  $\alpha$  subunit of eukaryotic translation initiation factor 2 (eIF-2 $\alpha$ ), leading to a decrease in the rate of initiation of translation. Assays using purified PKR and purified eIF2 demonstrated that the NS1 protein blocks the dsRNA activation of PKR, and experiments using reticulocyte extracts showed that the NS1 protein blocks the inhibition of translation caused by dsRNA activation of PKR. The implications of these results for control mechanisms occurring in influenza virus-infected cells are discussed. © 1995 Academic Press, Inc.

### INTRODUCTION

The NS1 protein of influenza A virus is a small non-structural RNA-binding protein (Qiu and Krug, 1993; Qiu *et al.*, 1995). Like other RNA binding proteins, the NS1 protein regulates posttranscriptional steps in gene expression by interacting with specific RNA sequences. The NS1 protein has been shown to function in two post-transcriptional steps. First, this protein inhibits the nuclear export of poly(A)-containing mRNAs (Alonso-Caplen *et al.*, 1992; Fortes *et al.*, 1994; Qian *et al.*, 1994; Qiu and Krug, 1993). To carry out this function, the NS1 protein recognizes and binds to the 3' poly(A) sequence of mRNA (Qiu and Krug, 1994). In addition, the NS1 protein inhibits pre-mRNA splicing (Fortes *et al.*, 1994; Lu *et al.*, 1994; Qiu *et al.*, 1995). Unlike other proteins that regulate splicing, the NS1 protein binds to a key spliceosomal RNA, U6 small nuclear RNA (snRNA), to carry out its function (Qiu *et al.*, 1995). The binding site on U6 snRNA is a purine-containing bulge imbedded in a stem structure. In addition to an RNA-binding domain, the NS1 protein has a second functional domain, called an effector domain, that is presumed to interact with host cell nuclear proteins (Qian *et al.*, 1994).

Here we present evidence indicating that the NS1 protein regulates another posttranscriptional step. The NS1 protein also binds to double-stranded RNA (dsRNA) (Ha-

tada and Fukuda, 1992; present study) and as a consequence blocks the activation of the dsRNA-activated protein kinase (PKR) *in vitro* (present study). PKR phosphorylates the  $\alpha$  subunit of the eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) (Galabru and Hovanessian, 1987; Samuel, 1993). This phosphorylation has been shown to lead to a decrease in the rate of initiation of translation (reviewed in Hershey, 1991; Merrick, 1992; Rhoads, 1993). During initiation eIF2 forms a ternary complex with GTP and initiator met tRNA. After initiation is completed, eIF2 is released from the ribosomes in the form of eIF2-GDP. The exchange factor eIF2B then replaces the GDP associated with eIF2 with GTP, thereby regenerating the functional form of eIF2, namely eIF2-GTP. However, when the  $\alpha$  subunit of eIF2 is phosphorylated, this exchange does not occur and eIF2B is trapped in a stable complex with eIF2, causing a limitation in functional eIF2-GTP. Because virus infection can lead to the activation of PKR and the consequent reduction in the rate of protein synthesis, various viruses have evolved mechanisms to prevent PKR activation (reviewed in Katze, 1992; Mathews, 1993; Samuel, 1993). The present study indicates that one of the mechanisms employed by influenza virus likely involves the NS1 protein.

### MATERIALS AND METHODS

**RNA-binding assays.** NS1 wild-type and mutant proteins were expressed as glutathione S-transferase (GST)-NS1 fusions and were purified as previously de-

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scribed (Qiu and Krug, 1994). The NS1 protein was cleaved from the fusion by treatment with protease factor Xa. Double-stranded reovirus RNA (kindly provided by Aaron Shatkin) was labeled using T4 RNA ligase and [ $^{32}$ P]pCp (final specific activity of RNA, 6000 cpm/ng). For generating sense and antisense transcripts of the pGEM1 polylinker, the pGEM 1 plasmid was cut at *Eco*RI and *Hind*III sites and transcribed by both Sp6 and T7 RNA polymerases in the presence of [ $\alpha$ - $^{32}$ P]UTP. The transcripts (in 10 mM HEPES, pH 7.6; 50 mM NaCl) were heated at 90° for 2 min and were allowed to anneal to each other by slow cooling to room temperature.  $^{32}$ P-labeled U6 snRNA was synthesized as described previously (Qiu *et al.*, 1995). For the binding experiments, the indicated amount of the NS1 protein and the target RNA were incubated for 20 min on ice in 20  $\mu$ l binding reactions containing 43 mM Tris-HCl, pH 8.0, 50 mM KCl, 8% glycerol, 2.5 mM dithiothreitol, 50  $\mu$ g/ml *Escherichia coli* tRNA; and 0.5 units/ $\mu$ l RNasin (Qiu and Krug, 1994). The protein-RNA complexes were resolved from unbound RNA by electrophoresis on 6% nondenaturing polyacrylamide. The running buffer contained 50 mM Tris-borate, pH 8.0; 1 mM EDTA. Gels were electrophoresed at 150 V for 3 hr at 4° or, for the reovirus experiments, 350 V for 18 hr at 4°. The gels were then dried and autoradiographed.

**In vitro translation assays.** Nuclease-treated rabbit reticulocyte extracts were programmed with luciferase mRNA, which was usually generated *in situ* by T7 RNA polymerase transcription of the luciferase gene (TNT coupled reticulocyte system, Promega). The reaction mixtures contained [ $^{35}$ S]methionine, an amino acid mixture (minus methionine), T7 RNA polymerase, and DNA encoding luciferase mRNA, and translation was carried out for 60 min at 30°. The amount of luciferase synthesized was determined by gel electrophoresis on SDS-containing 12.5% polyacrylamide gels, followed by fluorography.

**Assay of PKR activation.** PKR was purified using an immunoaffinity column (Galabru and Hovanessian, 1987), and purified eIF2 was kindly provided by Leonard Jefferson. Prior to the activation assay, ds reovirus RNA (final concentration of 0.01  $\mu$ g/ml) was incubated with the indicated amount of wild-type NS1 protein or of the NS1 $\Delta$ 1 deletion mutant under activation conditions (Lee *et al.*, 1994) for 20 min at 30°. Two controls were run, one without both ds reovirus RNA and the NS1 protein and one without the NS1 protein but containing ds reovirus RNA. After this preincubation, purified PKR (1.3 ng), purified eIF-2 (60 ng), and [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol) were added, and the reaction mixtures were incubated for an additional 15 min at 30°. The reactions were terminated by the addition of the SDS-containing gel buffer, followed by boiling for 5 min. The phosphorylated proteins were

then analyzed by electrophoresis on a SDS-containing, 10% polyacrylamide gel.

## RESULTS

### The RNA-binding domain of the NS1 protein binds to double-stranded RNA

The NS1 protein binds to two RNA sequences, poly(A) and the stem-bulge in U6 snRNA, to carry out its functions in nuclear mRNA export and pre-mRNA splicing, respectively (Qiu and Krug, 1993; Qiu *et al.*, 1995). Both of these RNA sequences are highly ordered. The most highly ordered RNA sequence would be expected to be dsRNA. We determined whether the NS1 protein also bound specifically to dsRNA, particularly in light of a previous report that this was the case (Hatada and Fukuda, 1992). Authentic dsRNA, reovirus genomic RNA, labeled at the 3' end, was incubated with increasing concentrations of the NS1 protein, and the mixtures were analyzed by native gel electrophoresis (Fig. 1). In the presence of 0.05  $\mu$ M of the NS1 protein, almost all of the ds reovirus RNA segments were shifted to more slowly migrating RNA-protein complexes. As the NS1 protein concentration was increased further, the complexes increased in size, indicating that multiple NS1 proteins were binding to the ds reovirus RNAs. This NS1 protein titration curve is similar to that obtained previously with U6 snRNA (Qiu *et al.*, 1995).

To establish that the previously identified RNA binding domain of the NS1 protein (Qian *et al.*, 1994) was required for the binding of dsRNA, NS1 proteins with mutations in the RNA binding domain — NSm2 and NSm3 — were used in the gel shift assay. These mutant NS1 proteins do not bind to either poly(A) or U6 snRNA (Qian *et al.*, 1994; Lu *et al.*, 1994; Qiu *et al.*, 1995). Neither of the two mutant NS1 proteins (at the high concentration of 0.4  $\mu$ M) bound to ds reovirus RNA (Fig. 2A). The same results were obtained with another dsRNA, one that was generated by annealing the sense and antisense transcripts of the polylinker of the pGEM1 plasmid (Fig. 2B). This small dsRNA of 29 base pairs formed a single discrete RNA-protein complex when incubated with wild-type NS1 protein (0.4  $\mu$ M). In contrast, no detectable complex was formed in the presence of the same concentration of the NSm2, NSm3, or NSm4 RNA-binding mutant proteins, or the NS $\Delta$ 1 protein containing a deletion of the RNA-binding domain. These results indicate that the previously identified wild-type RNA-binding domain of the NS1 protein (Qian *et al.*, 1994) is required for binding to dsRNA as well as for binding to poly(A) and U6 snRNA.

Only dsRNA and not single-stranded RNA competed with a dsRNA target for the binding domain of the NS1 protein (Fig. 3A). Thus, unlabeled ds poly(I):(C) (1.5  $\mu$ g/ml or 0.6 nM) efficiently blocked the binding of the 29

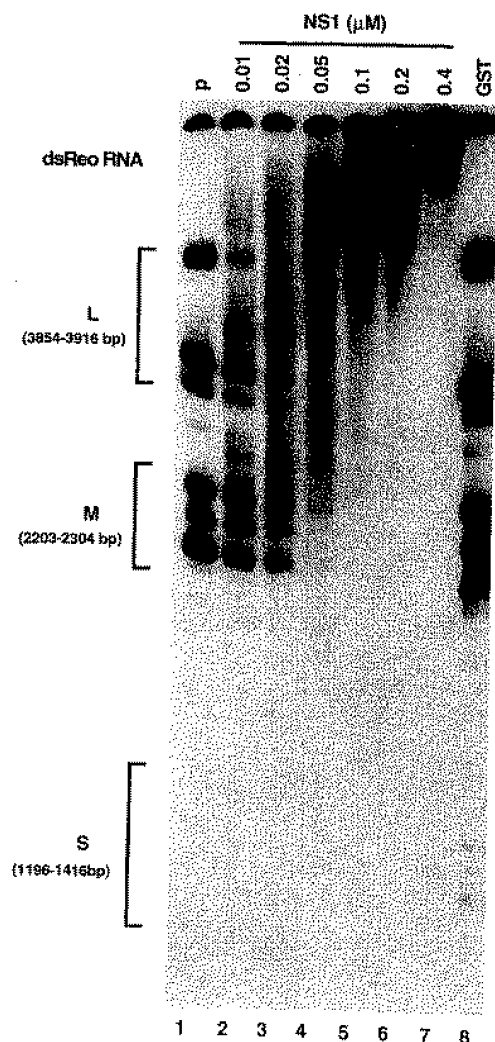


FIG. 1. The influenza virus NS1 protein binds to ds reovirus RNA.  $^{32}$ P-labeled ds reovirus RNA (16 ng, approximately 0.5 nM) was incubated with increasing concentrations of the NS1 protein (lanes 2–7), or with GST (0.4  $\mu$ M) (lane 8), and the mixtures were subjected to nondenaturing gel electrophoresis. Lane 1, P, reovirus RNA alone. L, M, S, large, medium, and small ds reovirus RNA segments, respectively.

base pair ds pGEM1 RNA (1.0 mM) to the NS1 protein (0.4  $\mu$ M) (lane 3), whereas the same concentration of unlabeled single-stranded poly(I) or poly(C) had no effect on the binding of ds pGEM1 RNA (lanes 4 and 5). Because each molecule of ds poly(I)-(C) contains about 65 times the number of base pairs as the ds pGEM1 RNA, it can be presumed that in this competition assay the number of binding sites in poly(I)-(C) at 0.6 mM was about 40 times that in ds pGEM1 RNA at 1.0 nM. Unlabeled ds RNA (reovirus RNA) also competed with a different labeled target RNA, U6 snRNA (at 1.0 nM), for binding to the NS1 protein (Fig. 3B). Because U6 snRNA most likely contains a single binding site for the NS1 protein (Qiu *et al.*, 1995), the lowest level of unlabeled ds reovirus RNA used in this competition assay (1.25  $\mu$ g/ml or about

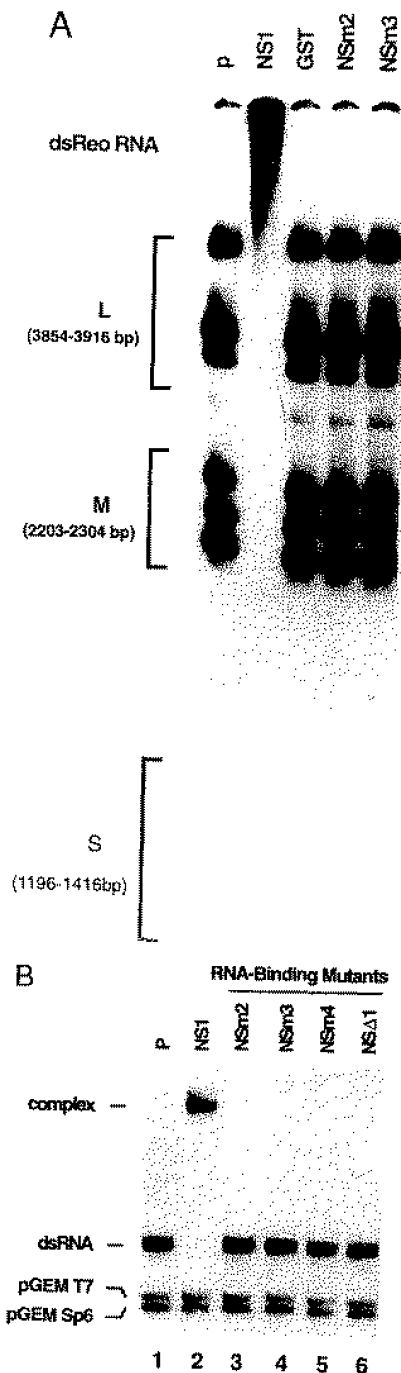


FIG. 2. Wild-type RNA-binding domain of the NS1 protein is required for binding to ds RNA. (A)  $^{32}$ P-labeled ds reovirus RNA (approximately 0.5 nM) was incubated with 0.4  $\mu$ M wild-type NS1 protein (lane 2), GST (lane 3), NSm2 mutant protein (lane 4), or NSm3 mutant protein (lane 5), and the mixtures were subject to nondenaturing gel electrophoresis. Lane 1, P, reovirus RNA alone. L, M, S, large, medium, and small ds reovirus RNA segments, respectively. (B)  $^{32}$ P-labeled ds pGEM1 RNA (1.0 nM) was incubated with 0.4  $\mu$ M wild-type NS1 protein (lane 2), NSm2 mutant protein (lane 3), NSm3 mutant protein (lane 4), NSm4 mutant proteins (lane 4), or NS $\Delta$ 1 deletion mutant protein (lane 5), and the mixtures were subjected to nondenaturing gel electrophoresis. Lane 1, P, ds pGEM1 RNA alone. pGEMT7 and pGEMSp6, the unannealed sense and antisense single-stranded RNAs.

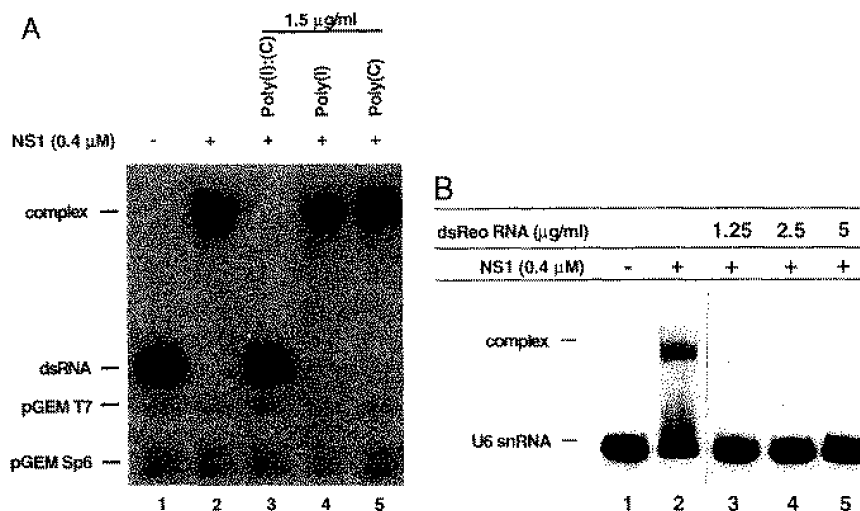


FIG. 3. Double-stranded RNA competes with both double-stranded RNA (A) and U6snRNA (B) for binding to the NS1 protein. (A)  $^{32}$ P-labeled ds pGEM1 RNA (1.0 nM) was mixed with 1.5  $\mu$ g/ml ds poly (I):(C) (approximately 2000 base pairs) (lane 3), single-stranded poly (I) (lane 4), or single-stranded poly (C), followed by the addition of 0.4  $\mu$ M NS1 protein. After incubation, the mixtures were subjected to nondenaturing gel electrophoresis. Lane 1, ds pGEM1 RNA in the absence of the NS1 protein. Lane 2, ds pGEM1 RNA incubated with the NS1 protein in the absence of a competitor RNA. (B)  $^{32}$ P-labeled U6 snRNA (1.0 nM) was mixed with the indicated concentrations of ds reovirus RNA (lanes 3–5), followed by the addition of 0.4  $\mu$ M NS1 protein. The mixtures were subjected to nondenaturing gel electrophoresis. Lane 1, U6 snRNA in the absence of the NS1 protein. Lane 2, U6 snRNA incubated with the NS1 protein in the absence of a competitor RNA.

0.6 nM) contained approximately 60 times more binding sites (assuming 30 base pairs per binding site) than in U6 snRNA at 1.0 nM. These results indicate that the NS1 protein binding sites for U6 snRNA and for ds RNA are the same or at least overlapping, consistent with the mutant data presented above.

#### The NS1 protein blocks the inhibition of translation caused by dsRNA

The addition of dsRNA to cell-free extracts has been shown to activate PKR which phosphorylates eIF-2 $\alpha$ , leading to the inhibition of protein synthesis (reviewed in Rhoads, 1993; Samuel, 1993). The effect of increasing concentrations of ds reovirus RNA on protein synthesis catalyzed by reticulocyte extracts is shown in Fig. 4A. Translation was programmed by luciferase mRNA generated by transcription during the translation reaction. The same results were obtained in all the translation experiments when luciferase mRNA was added directly to the translation reaction. Relative to the amount of translation in the absence of dsRNA (lane 7), the addition of increasing amounts of dsRNA, from 0.001 to 1.0  $\mu$ g/ml, caused increasing amounts of inhibition of translation (lanes 1–5). When the dsRNA concentration was increased to 10  $\mu$ g/ml, translation inhibition was slightly reversed (lane 6). Reversal of inhibition at high DNA concentrations is characteristic of PKR (Samuel, 1993).

Preincubation of the NS1 protein with ds reovirus RNA (0.1  $\mu$ g/ml) prevented the inhibition of translation caused by this dsRNA (Fig. 4B). Partial restoration of translation

occurred with 0.1  $\mu$ M NS1 protein, and complete restoration occurred with 0.2  $\mu$ M NS1 protein. Translation restoration required a wild-type RNA binding domain in the NS1 protein (Fig. 4C). NS1 proteins containing either a point mutation in the RNA-binding domain (NSm2) or a deletion of the RNA-binding domain (NS1 $\Delta$ 1) did not restore translation. The most efficient restoration of translation occurred when the NS1 protein was preincubated with ds reovirus RNA prior to the addition of the reticulocyte extract (Fig. 4D). When the NS1 protein was added at the same time as the reticulocyte extract, the restoration was considerably reduced. No detectable restoration occurred when the NS1 protein was added 5 min after the reticulocyte extract. These results indicate that the NS1 protein, by binding to dsRNA via its RNA-binding domain, most likely blocks the activation of PKR and the subsequent inhibition of translation. Preincubation of the dsRNA with the NS1 protein would enable all the dsRNA to be sequestered in complexes with the NS1 protein prior to the introduction of the unactivated PKR present in the reticulocyte extract.

#### The NS1 protein blocks the activation of PKR

To prove directly that the NS1 protein blocked the activation of PKR, we determined the effect of this protein on the dsRNA activated phosphorylation of purified PKR and its purified substrate eIF2 $\alpha$  (Fig. 5). The phosphate donor was [ $\gamma$ - $^{32}$ P]ATP. In the absence of ds reovirus RNA, neither PKR nor the eIF2 $\alpha$  substrate was phosphorylated (lane 1). The addition of a low level of ds reovirus RNA

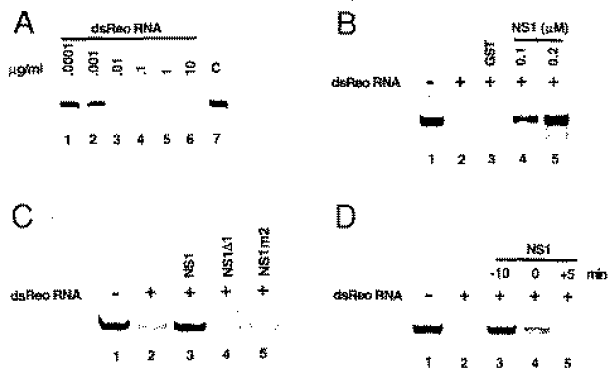


FIG. 4. The NS1 protein blocks the inhibition of translation caused by ds RNA. (A) Increasing concentrations of ds reovirus RNA were mixed with 5  $\mu$ l of nuclease-treated reticulocyte extracts (lanes 1–6) and incubated for 10 min at 30°. As a control (c) the extract was incubated in the absence of ds reovirus RNA (lane 7). The reaction mixtures were then supplemented with translation components as described under Materials and Methods. After incubation for 60 min at 30°, the proteins were analyzed by SDS gel electrophoresis. (B) Reovirus RNA (0.1  $\mu$ g/ml) was incubated with 0.2  $\mu$ M GST (lane 3), 0.1  $\mu$ M NS1 protein (lane 4), or 0.2  $\mu$ M NS1 protein (lane 5) for 10 min at 30°. As a control, no protein was added to the reovirus RNA (lane 2). Reticulocyte extract was added for another incubation at 30° for 10 min. These mixtures were then supplemented with the translation components, and translation was carried out for 60 min at 30°. Lane 1, no ds reovirus RNA was added to the reticulocyte extract. (C) Reovirus RNA (0.1  $\mu$ g/ml) was incubated with 0.2  $\mu$ M wild-type NS1 protein (lane 3), NS1 $\Delta$ 1 deletion mutant protein (lane 4), or NS1m2 mutant protein (lane 5) for 10 min at 30°. As a control, no protein was added to the reovirus RNA (lane 2). This was followed sequentially by a 10-min incubation at 30° with reticulocyte extract and then a translation reaction for 60 min at 30°. Lane 1, no ds reovirus RNA was added to the reticulocyte extract. (D) The NS1 protein (0.2  $\mu$ M) was either preincubated for 10 min with the reovirus RNA (0.1  $\mu$ g/ml) as described above (lane 3), added to the reticulocyte extract at the same time as the reovirus RNA (lane 4), or 5 min after the reovirus RNA (lane 5). As a control, no protein was added to the reovirus RNA (lane 2). After a total of 10 min incubation with the reticulocyte extract, the translation components were added for a subsequent incubation for 60 min at 30°. Lane 1, no ds reovirus RNA added to the reticulocyte extract.

(0.01  $\mu$ g/ml) caused a dramatic stimulation of the phosphorylation of both PKR and eIF2 $\alpha$  (lane 2). Preincubation of the ds reovirus RNA with wild-type NS1 protein completely blocked the phosphorylation of both PKR and eIF2 $\alpha$  (lanes 3–5). In contrast, the NS1 protein containing a deletion of the RNA-binding domain (NS1 $\Delta$ 1) did not inhibit the phosphorylation of PKR and eIF2 $\alpha$  (lanes 6–8). These results demonstrate directly that the NS1 protein blocks the dsRNA activation of PKR. As a consequence this kinase does not autophosphorylate and does not phosphorylate eIF2 $\alpha$ .

## DISCUSSION

The influenza virus NS1 protein has two functional domains, an RNA-binding domain and an effector domain that is presumed to interact with host cell nuclear pro-

teins (Qian *et al.*, 1994). The NS1 protein RNA-binding domain has now been shown to bind to three different RNAs, poly(A), a stem-bulge region in U6 snRNA, and double-stranded RNA (Hatada and Fukuda, 1992; Qiu and Krug, 1993; Qiu *et al.*, 1995; present study). Mutations in the NS1 RNA-binding domain eliminate binding activity with all three RNAs, and the three RNAs compete with each other for binding to the NS1 protein (Qian *et al.*, 1994; Lu *et al.*, 1994; Qiu *et al.*, 1995; present study), indicating that the same domain of the protein likely binds to all three RNAs. Are there common features in these three RNAs that are recognized by the RNA-binding domain of the NS1 protein? One possibility is that structures similar to the A form of dsRNA are present in both poly(A) and the stem-bulge of U6 snRNA, and that these structures are recognized by the NS1 protein. To elucidate how the NS1 protein recognizes and binds to its RNA targets, we have initiated structural studies of the NS1 protein and of its complex with a target RNA. It is likely that the mode of binding of the NS1 protein to its RNA targets will differ from that of previously reported ds RNA-binding proteins, because the NS1 protein RNA-binding domain does not share any significant homology with the RNA-binding domains of these other ds RNA-binding proteins (St. Johnston *et al.*, 1992; Green and Mathews, 1992).

Previous studies have indicated that during influenza virus infection PKR is first activated by viral ds RNAs (Katze, 1992). Subsequently PKR is suppressed by processes requiring viral gene expression, thereby maintaining high levels of protein synthesis. A 58-kDa cellular protein, which was purified from virus-infected cells, has been shown to inhibit the phosphorylation of eIF2 $\alpha$  by

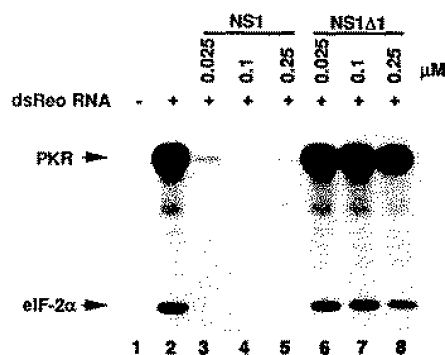


FIG. 5. The NS1 protein blocks the activation of PKR. Double-stranded reovirus RNA (0.01  $\mu$ g/ml) was incubated for 20 min at 30° in the absence of added protein (lane 2), in the presence of the indicated concentration of wild-type NS1 protein (lanes 3–5), or in the presence of the indicated concentration of the NS1 $\Delta$ 1 deletion mutant protein (lanes 6–8). Purified PKR and eIF-2, and [ $\gamma$ - $^{32}$ P]ATP were added and the reaction mixtures were then incubated for 15 min at 30°. As a control, neither ds reovirus RNA nor the NS1 protein was added to the PKR activation reaction (lane 1). Phosphorylated proteins were resolved by gel electrophoresis.

PKR, including already activated PKR (Lee *et al.*, 1990, 1994). Evidence has been presented that the virus activates the 58-kDa cellular protein by dissociating it from an inhibitory cellular factor (Lee *et al.*, 1992). The results in the present report indicate that there is likely a second mechanism for inhibiting PKR, namely blocking activation of this kinase by sequestering viral dsRNAs in complexes with the viral NS1 protein. Our *in vitro* results show that the NS1 protein binds to dsRNA and that this binding blocks the dsRNA from activating the PKR kinase. As a consequence, eIF-2 $\alpha$  was not phosphorylated and protein synthesis was not inhibited. Based on these results our working hypothesis is that in infected cells the NS1 protein, which is predominantly in the nucleus (Lazarowitz *et al.*, 1971; Compans, 1973; Krug and Etkind, 1973), sequesters viral dsRNAs in the nucleus, where these molecules would be expected to be generated as a by-product of viral RNA transcription and replication (Krug *et al.*, 1989). This would block the dsRNA activation of PKR in the cytoplasm. If the NS1 protein does carry out this function in infected cells, then influenza virus would likely mount a two pronged attack against PKR, blocking the activation of the kinase and inhibiting the activity of any kinase that happens to be activated (Lee *et al.*, 1990, 1992; present study). Vaccinia virus also apparently utilizes an analogous two pronged attack, a dsRNA-binding protein that prevents activation of the kinase and a viral protein that functions as a pseudosubstrate in place of eIF2 $\alpha$  for any kinase that happens to be activated (Akkaraju *et al.*, 1989; Beattie *et al.*, 1991; Watson *et al.*, 1991; Beattie *et al.*, 1995). On the other hand, reovirus appears to employ only a dsRNA-binding protein, the  $\sigma$ 3 protein, to block the activation of the kinase (Lloyd and Shatkin, 1992).

Our results indicate that the NS1 protein most likely enhances the rate of initiation of translation in infected cells by blocking the activation of PKR. Does the NS1 protein have other effects on translation? A small fraction of the NS1 protein in infected cells appears to be associated with polyribosomes (Compans, 1973; Krug and Etkind, 1973), and it is conceivable that this NS1 protein population could be binding to one or more polyribosome-associated RNAs. In addition, two groups have reported that the NS1 protein stimulated the translation of either a few specific viral mRNAs (Enami *et al.*, 1994) or many viral mRNAs (Luna *et al.*, 1995), and that this stimulation apparently required, at least in part, sequences in the 5' untranslated regions of the viral mRNAs. It can be argued that these effects on translation could result from the activity of the NS1 protein in blocking the activation of PKR. Both of the studies cited above used transfection experiments, which have been shown to result in the activation of PKR that is directed against only the plasmid-derived mRNAs (Kaufman and Murtha, 1987). The NS1 protein would be expected to

block this activation, thereby increasing the rate of initiation of translation of the plasmid-derived mRNAs. In fact, one of the studies cited above (Luna *et al.*, 1995) showed that a major effect of the NS1 protein was to increase the rate of translation initiation of the plasmid-derived mRNAs in the transfected cells. The blockage of PKR activation by the NS1 protein would be expected to favor the translation of viral mRNAs versus cellular mRNAs if this blockage were partial rather than complete. In this situation some phosphorylation of eIF2 $\alpha$  would continue to occur, but the amount of phosphorylated eIF2 $\alpha$  would not exceed the amount of the eIF-2B recycling factor. Consequently, translation initiation would not cease, but rather would continue at a reduced rate. Under these conditions those mRNAs that are better initiators of translation ("better mRNAs") would be predicted to interact preferentially with the limiting amount of eIF-2-containing initiation complexes on ribosomes (Lodish, 1974, 1976). In fact, we most likely achieved these very conditions in earlier experiments, and demonstrated that under these conditions influenza viral mRNAs behaved as "better mRNAs," i.e., they were translated preferentially versus adenovirus and cellular mRNAs (Katze *et al.*, 1984, 1986). Further experiments are needed to clarify the precise role of the NS1 protein in translation in infected cells.

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# Binding of the Influenza Virus NS1 Protein to Double-Stranded RNA Inhibits the Activation of the Protein Kinase That Phosphorylates the eIF-2 Translation Initiation Factor

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The NS1 protein of influenza A virus binds not only to poly(A) and a stem-bulge region in U6 small nuclear RNA (snRNA), but also to double-stranded (ds) RNA. Binding assays with NS1 protein mutants established that the previously identified RNA-binding domain of the NS1 protein is required for binding to ds RNA as well as for binding to poly(A) and U6 snRNA. In addition, dsRNA competed with U6 snRNA for binding to the NS1 protein, consistent with both RNAs sharing the same binding site on the protein. As a consequence of its binding to dsRNA, the NS1 protein blocks the activation of the dsRNA-activated protein kinase (PKR) *in vitro*. This kinase phosphorylates the  $\alpha$  subunit of eukaryotic translation initiation factor 2 (eIF-2 $\alpha$ ), leading to a decrease in the rate of initiation of translation. Assays using purified PKR and purified eIF2 demonstrated that the NS1 protein blocks the dsRNA activation of PKR, and experiments using reticulocyte extracts showed that the NS1 protein blocks the inhibition of translation caused by dsRNA activation of PKR. The implications of these results for control mechanisms occurring in influenza virus-infected cells are discussed. © 1995 Academic Press, Inc.

## INTRODUCTION

The NS1 protein of influenza A virus is a small non-structural RNA-binding protein (Qiu and Krug, 1993; Qiu et al., 1995). Like other RNA binding proteins, the NS1 protein regulates posttranscriptional steps in gene expression by interacting with specific RNA sequences. The NS1 protein has been shown to function in two posttranscriptional steps. First, this protein inhibits the nuclear export of poly(A)-containing mRNAs (Alonso-Caplen et al., 1992; Fortes et al., 1994; Qian et al., 1994; Qiu and Krug, 1995). To carry out this function, the NS1 protein recognizes and binds to the 3' poly(A) sequence of mRNA and Krug, 1994). In addition, the NS1 protein inhibits pre-mRNA splicing (Fortes et al., 1994; Lu et al., 1994; Qiu et al., 1995). Unlike other proteins that regulate splicing, the NS1 protein binds to a key spliceosomal RNA, small nuclear RNA (snRNA), to carry out its function (Lu et al., 1995). The binding site on U6 snRNA is a stem-containing bulge imbedded in a stem structure. In addition to an RNA-binding domain, the NS1 protein has a second functional domain, called an effector domain, that is presumed to interact with host cell nuclear proteins (Qian et al., 1994).

In the present study, we present evidence indicating that the NS1 protein also regulates another posttranscriptional step. The NS1 protein also binds to double-stranded RNA (dsRNA) (Hata-

tada and Fukuda, 1992; present study) and as a consequence blocks the activation of the dsRNA-activated protein kinase (PKR) *in vitro* (present study). PKR phosphorylates the  $\alpha$  subunit of the eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) (Galabru and Hovanessian, 1987; Samuel, 1993). This phosphorylation has been shown to lead to a decrease in the rate of initiation of translation (reviewed in Hershey, 1991; Merrick, 1992; Rhoads, 1993). During initiation eIF2 forms a ternary complex with GTP and initiator met tRNA. After initiation is completed, eIF2 is released from the ribosomes in the form of eIF2-GDP. The exchange factor eIF2B then replaces the GDP associated with eIF2 with GTP, thereby regenerating the functional form of eIF2, namely eIF2-GTP. However, when the  $\alpha$  subunit of eIF2 is phosphorylated, this exchange does not occur and eIF2B is trapped in a stable complex with eIF2, causing a limitation in functional eIF2-GTP. Because virus infection can lead to the activation of PKR and the consequent reduction in the rate of protein synthesis, various viruses have evolved mechanisms to prevent PKR activation (reviewed in Katze, 1992; Mathews, 1993; Samuel, 1993). The present study indicates that one of the mechanisms employed by influenza virus likely involves the NS1 protein.

## MATERIALS AND METHODS

**RNA-binding assays.** NS1 wild-type and mutant proteins were expressed as glutathione *S*-transferase (GST)-NS1 fusions and were purified as previously de-

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